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Vaccination of dams before pregnancy has a limited effect on the amount but no effect on the presence of porcine circovirus type 2 (PCV2) DNA in fetal tissues

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1. Summary

Infection of pigs with PCV2 is associated with several diseases. The onset of the viral infection is not clear but may occur in utero and may be prevented by vaccination of dams. In this study we determined the prevalence and load of PCV2 protein and DNA in tissues of fetuses obtained from non-vaccinated or vaccinated healthy dams. Also, sera from these piglets and from the dams were collected for PCV2-specific antibody determination. We analysed 91 fetuses of 7 naïve dams, 58 fetuses of 4 dams vaccinated with Ingelvac® CircoFLEX™ and 94 fetuses of 6 dams vaccinated with Circovac®. In none of the fetal tissues macroscopic or histopathological alterations were found. All tissues investigated randomly by immunohistochemistry for the presence of PCV2 protein were negative. To detect viral DNA, various tissues were analysed by fluorescence in situ hybridisation. All thymi, 91% of the mesenteric lymph nodes, 60% of the Peyer's patches of the ileum and 24% of the spleen were PCV2 DNA positive. Fetuses of some vaccinated dams had a significantly lower PCV2 DNA load. All fetal sera were negative for anti-PCV2 IgG and IgM antibodies whereas sera from 13 dams tested contained anti-PCV2 IgG antibodies. Sera of 16 dams were negative for anti-PCV2 IgM antibodies indicating recent infection. In none of the fetal tissues analysed PCV2 specific proteins were detected, whereas at least on fetal tissue was positive for PCV2 DNA irrespective of the vaccination status of the dam. Hence, the viral load but not the prevalence of viral infection may be influenced by vaccination.

2. Introduction

Infections with porcine circovirus type 2 (PCV2) can cause several disease manifestations in pigs, named porcine circovirus associated disease (PCVAD) which includes postweaning multisystemic wasting syndrome (PMWS), porcine dermatitis and nephritis syndrome (PDNS), porcine respiratory disease complex (PRDC), proliferative necrotising pneumonia (PNP), granulomatos enteritis in weaned pigs, necrotising lymphadenitis and also reproduction problems [1]. PMWS was first described by Harding and Clark in 1997 [2]. Since then, PMWS has emerged worldwide and is one of the economically most important swine diseases [3, 4].

PCV2-associated reproductive failure in dams was first reported in 1999 [5] and since then repeatedly described by other research groups [6-8]. Clinical manifestations of reproductive failure vary among farms and gestation stage including mummification, abortion, stillborn or weak-born piglets, delayed farrowing [6] and pseudopregnancy [9]. Reproductive disorders are more often observed in gilts and newly established herds [5, 7, 9]. Pathological findings in PCV2 infected fetuses can show gross lesions as pulmonary edema, hepatomegaly and ascites as well as histological myocardial necrosis and fibrosis [5]. According to Segalés et al. [10], diagnosis of PCV2-associated fertility disorders is based on the fulfilment of three criteria: (I) clinical signs such as late-term abortions and increased numbers of stillbirths, mummified fetuses or weak-born piglets, (II) microscopical lesions such as myocardial lesions like fibrosing and/or necrotising myocarditis and (III) the presence of plenty of PCV2 antigen or DNA in the lesions.

Particular high concentrations of PCV2 antigen are readily visible in dead fetuses by immunohistochemistry (IHC) [5, 7, 8, 11, 12]. However, in healthy viable neonatal piglets PCV2 infection can also be demonstrated by either PCR and/or serum anti-PCV2 antibodies. Interestingly, Shen et al. [13] found DNA of PCV2 in serum of newborn piglets in 40% before colostrum intake and 21.4% were positive for anti-PCV2 IgG antibodies.

Horizontal PCV2 infection is thought to be the main route of virus transmission [14, 15]. However, there are also reports about vertical transmission from the parents to progeny either through the placental barrier [5, 12], through a pre-existing infection of the dam's reproductive tract [16] or via an infection of gametes, mainly through semen [17].

Vaccination against PCV2 almost eliminated the occurrence of PCVAD and improves mortality rates of nursery and fattening pigs as well as pig performance in farms, chronically infected with PCV2 [18]. Although vaccination against PCV2 reduces viremia, PCV2 DNA was still detected in colostrum and piglets from vaccinated dams by sensitive PCR [19].

In Switzerland reproduction failure due to PCV2-infection was first described in 2008 [20]. However, a prevalence-study of 286 fetuses of 113 dams from 59 farms with anamnestically increased reproductive disorders, before nationwide vaccination against PCV2, revealed only 4% PCV2-associated fertility disorders, fulfilling the case definition criteria, in particular a high amount of PCV2 antigen visible by IHC [11]. However, by PCR and fluorescence in situ hybridisation (FISH) most fetuses harboured PCV2 DNA in small amounts (manuscript in preparation). In this study thymus was found to be the organ with the highest and most abundant PCV2 infections. Thus, we have evidence that fetal infections are more common than originally thought, even though most fetuses are inconspicuous by anti-PCV2 IHC.

Despite thymus atrophy is a common lesion in PMWS, thymus involvement in PCV2 infected piglets and fetuses is only sporadically mentioned in literature [21-26]. Interestingly, chicken anemia virus (CAV), a *Gyrovirus*, which belongs to the family of *Circoviridae* like PCV2, is also transmitted vertically independent of the antibody status in chicks [27]. Both are single stranded DNA viruses with a double stranded DNA isoform during viral replication [28]. CAV DNA could be detected in blastodisks and semen of antibody positive and negative chickens [27]. The lymphoid organs and gonads had the highest incidence of CAV DNA. In young chickens thymus atrophy occurs analogous to PMWS [29].

The aims of this study were first to research high prevalence probability of PCV2 infected fetuses from slaughter dams in late undisturbed pregnancy with special attention to primary (thymus) and secondary lymphatic organs and second to investigate the effect of dam vaccination on the occurrence of fetal infections with PCV2.

3. Materials and methods

3.1. Animals and herds

243 fetuses of 17 dams with undisturbed late pregnancy (dams were in 2.-11. gestation) from 7 different breeding farms without a farm history of increased reproductive problems were investigated in this study. Dams of different gestation stages (77–109 th) of 7 farms without PCV2 related problems were purchased and immediately after slaughter hysterectomized. Our study was carried out according to Swiss Animal Welfare guidelines (study number 202/2010). 5 dams were naturally and 12 were artificially inseminated. The control group included 91 fetuses of 7 dams from 3 different farms without PCV2 vaccination. 58 fetuses of 4 dams of 2 different farms were vaccinated with Ingelvac® CircoFLEX™ (baculovirus expressed subunit vaccine, Boehringer Ingelheim GmbH) 1 to 4 weeks before insemination (a single shot vaccine) and these fetuses were accounted to the FLEX-group. One dam in the FLEX-group was immunized just before every insemination. The CVac-group included 94 fetuses of 6 dams from 3 different farms vaccinated with Circovac® (inactivated PCV2-vaccine with paraffin adjuvans, MERIAL) with a basic immunisation before first mating and a booster between the 50th and the 95th day of gestation. For further analysis, we splitted the FLEX-group and the CVac-group into the SVac-group, where dams were vaccinated once, and into the MVac-group, where dams received more than one immunization. In every farm, except for one, the piglets were additionally vaccinated against PCV2 either with Ingelvac® CircoFLEX™ or Circovac® at about 3 weeks of age. Table 1 shows a summary of litter characteristics and reproduction parameters of the groups.

Table 1: Experimental groups, animals and management of reproduction

Groups	Dam	Farm	Vaccination	Management	Litter	Gestation	Number of fetuses	Mummified fetuses
Control	1	4	None	natural breeding	2	104	10	2
	2	1	"	insemination	5	98	10	0
	3	1	"	"	6	99	10	0
	4	2	"	"	5	102	20	0
	5	2	"	"	3	102	15	0
	6	2	"	"	4	101	14	0
	11	4	"	natural breeding	1	77	12	2
FLEX	7	2	SVac[a]	insemination	7	99	16	1
	8	2	"	"	6	99	16	1
	9	2	"	"	9	100	13	0
	10	3	MVac[b]	"	7	107	13	3
CVac	12	5	MVac	insemination	10	104	8	1
	13	5	"	"	6	98	17	0
	14	6	"	"	6	109	12	0
	15	6	"	natural breeding	7	109	19	0
	16	7	"	"	5	104	21	0
	17	7	"	"	6	105	17	0

Experimental groups, dam and farm identification number, management of reproduction, number of previous litters of dams, gestation (days), number and mummified fetuses are shown. For the grouping of FLEX and CVac see material and methods.

[a] SVac: single vaccination

[b] MVac: multiple vaccinations

3.2. Tissue and blood sampling

Serum and blood samples were individually collected from dams and fetuses. Fetuses were taken out of the gravid uteruses and blood was collected immediately by heart puncture. Serum samples were centrifuged at 3600g for 10 minutes and then all blood and serum samples were stored at -20°C until examination. Tissue samples from thymus, spleen, mesenteric lymph nodes, ileum, heart, liver, lung, bone marrow and placenta were collected at necropsy and fixed in 4% formalin for histological examination and for FISH evaluation of thymus, spleen, mesenteric lymph nodes and

Peyer's patches of the ileum. Also IHC was performed on the same tissue samples from randomly selected fetuses.

3.3. Histopathology

The formalin fixed tissues were embedded in paraffin wax one day after sampling. Sections of 2-3 µm were stained with hematoxylin and eosin by standard methods. All the collected organs were evaluated for the presence of histological lesions.

3.4. Fluorescence in situ hybridisation (FISH)

Tissue samples of thymus, spleen, mesenteric lymph nodes and ileum were fixed and paraffin-embedded. We used the DISCOVERY instrument (Ventana Medical System) for staining the slides and the experimental protocol was run with help of the DISCOVERY software.
In short, the slides were first deparaffinised with EZ Prep (Ventana Medical System) for 28 min and pre-treated for 4 min with Protease I (Ventana Medical System) at 37°C. Tissue sections were overlaid with corresponding 5' and 3' fluorescents labeled oligonucleotide probes after denaturizing for 4 min at 90°C. Hybridization lasted for 2 h at 37°C. Slides were washed in two stringency washes at 47°C for 4 min. Afterwards samples were treated with a 4% fixative reagent for 24 min to fix oligonucleotide probes to the target DNA. After two washing steps tissue sections were counterstained. Finally, slides were washed and mounted with FloureGuard Mounting Medium (Scy Tec). In every run we carried along a slide with a positive and a negative control.
For signal detection we used a Leica DMLB IF fluorescence microscope. Pictures were taken by Bonn Tec Imaging. To estimate the signal detection in the organs we used a score from 0 (negative, no cells were positively labeled), 1 (a very small number were positive labeled), 2 (slightly positive labeled cells), 3 (moderately positive labeled cells) to 4 (profoundly positive labeled cells).
PCV2 infected cells in thymus were arranged either in groups or solitarily distributed. Therefore we counted positive cells which occurred in groups or singly and compared the distribution between the groups.

To make the infection intensity of the four organs over all fetuses visible we used a score system from 0 to 4 based on the scale described above.
To differentiate between ssDNA and dsDNA we used the experimental protocol as described in Khaiseb et al. [30]. For signal detection a Leica SP5 Mid UV-VIS laser confocal scanning microscope at the Center for Microscopy and Imaris 7.3 image analysis software (University of Zurich, Switzerland) was used.

3.5. Immunohistochemistry (IHC)

From fixed and paraffin-embedded tissue we randomly selected 20 fetuses with highly infected thymus (score 3 or 4) determined with FISH and all mummified fetuses. We used the F217 monoclonal anti-PCV2 antibody for immunohistochemistry [31]. Tissue sections were first deparaffinised and incubated with pronase (S2019, Dako, Glostrup, Denmark) for 60 min, then rinsed with Tris-buffered saline containing Tween-20 (TBS/T20) (Wash Buffer 10x, S3006, DakoCytomation) and F217 monoclonal antibody supernatant was diluted 1:2 and then incubated for 60 min with tissue sections. After neutralizing the endogenous peroxidase activity (Peroxidase-Blocking Solution, S2023, DakoCytomation), the slides were washed with TBS/T20 and incubated with secondary dextran-bound peroxidase-conjugated goat anti-mouse/rabbit antibody (Envision-Kit Mouse K500, DakoCytomation) for 30 min. 10 min of substrate (amino-ethyl-carbazol (DakoCytomation)) conversion was given to detect specific antigen-antibody reaction. Afterwards, tissue sections were counterstained with haematoxylin. The immunohistochemical reactions were carried out in the DakoCytomation Autostainer at room temperature.

3.6. Serological examinations

A competitive enzyme-linked immunosorbent assay (SERELISA® PCV2b Ab Mono Blocking, SYNBIOTICS EUROPE SAS 2, Lyon, France) was used for anti-PCV2 IgG antibody detection according to manufacturer's instructions and a published reference [32]. Additionally, we supplemented the ELISA assay with internal controls, an additional positive and negative control serum to check the homogeneity of the plate

antigen coating as well as another known pig serum on each plate to normalize S-values amongst individual plates [18]. For calculation we used a ready-to-use Excel-sheet provided by SYNBIOTICS EUROPE SAS. We evaluated every S-value higher than 2000 EU as a positive result (internal experience).

Anti-PCV2 IgM antibodies were measured using INGEZIM Circovirus IgG/IgM (Ingenasa, Madrid), a captured immunoenzymatic assay specific for IgM antibody detection to PCV2. As in the manufacturer's instruction written we interpreted an O.D. higher than 0.7 as positive and an O.D. lower than 0.3 as negative.

3.7. Real-time PCR made visible with SYBR Green

We used a real-time PCR based SYBR Green method to determine blood PCV2 DNA template concentrations of dams and fetuses. DNA was extracted with the help of MagNA Pure LC DNA Isolation Kit II (Tissue) (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany, Version December 2005). In each separate PCR run we used predefined 5 different 10 PCV2 DNA template concentration increases of recombinant PCV2 sequence. In the real-time PCR we used a master mix supplemented for each reaction with 12.5 µl Sybr Green 2x and 20 µM oligonucleotides (PCV2 F, 5'-CGY TGG AGA AGG AAA AAY GGC and PCV2 R, 5'-GTA GTA TTC AAA GGG YAY AGA G). The real-time PCR was run on an ABI Fast 7500. Cycling parameters were 50°C for 2 min, 95°C for 10 min, 50 cycles of 95°C for 15 s, 62°C for 30 s and 65°C for 45 s followed by PCR reaction product dissociation at 95°C for 15 s, 60°C for 1 min and 95°C for 15 s. As negative controls several PBS, bovine serum and water samples were carried along each PCR run. PCV2 positive samples dissociation parameters were in the range of 78.3°C to 81.7°C.

3.8. PPV-diagnostic

In order to exclude a porcine parvovirus (PPV) infection a tissue homogenate from mummified fetuses, consisting of heart, lung, liver and kidney, underwent an examination by real-time PCR [33] or by indirect immunofluorescence (IIF) [34] depending on the crown-rump length of the fetus.

3.9. Statistical analysis

The R statistical software environment (R Core Team 2012) was used for all data modeling. Due to the potential for correlated observations (multiple observations from within the same dam, and dams nested within farms) generalized linear mixed models were utilized using the lme4 and nlme extensions packages for R. Akaikes Information Criterion (AIC) was used to choose an optimal form of random effects to account for correlations between observations. The chosen form of statistical model was then used to estimate the statistical significance of the effect of factors of interest, such as type and frequency of vaccination, through a standard likelihood-ratio test between the different groups. P-value of ≤ 0.05 was accepted as statistically significant.

4. Results

4.1. Clinical examination, reproduction parameters of dams and post-mortem examination of fetuses

All sows were clinically healthy at the time of slaughter. Necropsied fetuses had no macroscopic or histological lesions. Four mummified fetuses were found in two dams of the control group, five mummified fetuses from three different sows of two different farms and one mummified fetus were found in the FLEX- and CVac-group, respectively. All mummified fetuses were tested negative for PPV. Based on earlier results from IHC negative aborted fetuses (manuscript in preparation) we assume that all fetuses without lesions were PCV2 antigen negative. To confirm this hyothesis, we tested 20 randomly selected fetuses. All mummified fetuses as well as the 20 selected fetuses were tested negative for PCV2 antigen by IHC.

4.2. PCV2 antibodies in fetuses and dams

All examined fetuses had no detectable anti-PCV2 IgG and IgM antibodies. In the control group four sows had positive anti-PCV2 IgG antibody titers and three sows were negative for anti-PCV2 IgG antibodies (<2000 EU). The mean antibody titer in the control group was 8'784 EU. In the FLEX-group all dams had positive IgG antibody titers with an average titer of 57'505 EU and in the CVac-group five from six sows had positive anti-PCV2 IgG antibody titers with a mean value of 129'115 EU. However, one sow was repeatedly negative for anti-PCV2 antibodies. The average antibody titer from the SVac-group was 66'067 EU in comparison to the mean antibody titer of 115'215 EU from the MVac-group.

All sows of the CVac-group, except one, with a repeatedly, doubtfully positive result, were negative for anti-PCV2 IgM antibodies. Table 2 summarizes the results for anti-PCV2 IgG and IgM antibodies.

Table 2: PCV2-specific serum antibody and PCV2 PCR results.

Groups	Dam	Dam IgG	Fetal IgG	Dam IgM	Fetal IgM	Dam PCR	Fetal PCR
Control	1	1	0/10	-	0/10	n.d.[a]	0/10
	2	4846	0/10	-	0/10	-	1/10
	3	384	0/10	-	0/9	-	0/10
	4	17191	0/20	-	0/20	-	0/20
	5	5573	0/15	-	0/15	-	0/15
	6	33452	0/14	-	0/14	-	1/14
	11	43	0/12	-	0/12	-	0/12
FLEX	7	70055	0/16	-	0/16	-	0/16
	8	40571	0/16	-	0/16	-	0/16
	9	87576	0/13	-	0/13	-	0/13
	10	31819	0/13	-	0/13	-	0/13
CVac	12	950	0/8	-	0/8	-	0/8
	13	494438	0/17	-	0/17	+	1/17
	14	3031	0/12	-	0/12	-	0/11
	15	148331	0/19	-	0/19	-	1/19
	16	84459	0/21	+/-	0/21	-	1/21
	17	43480	0/17	-	0/17	-	2/17

Serum IgG antibody titers are shown as ELISA units, whereas fetal IgG and IgM specific to PCV2 and the PCR results are the numbers of positive results/group. All PCRs performed with sera resulted rarely and in slightly positive amplifications.

[a]n.d.: not done

4.3. PCV2 viremia in fetuses and dams

Only in a few fetuses (2.9%) low concentrations of PCV2 DNA were detected in fetal blood by real-time PCR. Namely, in 2 fetuses from two different sows of the control group and in 5 fetuses from 4 different sows of the CVac-group. All fetuses from the FLEX-group had no detectable PCV2 DNA (table 2). The concentrations of PCV2 DNA in fetal blood ranged from $1.5x10^5$ genome copies/ml to $6.5x10^5$ genome copies/ml. Only in one sow of the CVac-group $1.3x10^5$ genome copies/ml of PCV2 were detected. Blood from one sow in the control group was not available (table 2).

4.4. Prevalence of PCV2 infection and comparison of virus load in thymus, spleen, mesenteric lymph nodes and Peyer's patches of the ileum evaluated by FISH

PCV2 infection prevalence of fetal organs was as following: 242 of 242 examined fetuses (100% prevalence) contained PCV2 DNA containing cells in the thymus. Thymus of one fetus was not available. 217 of 238 examined mesenteric lymph nodes (91% prevalence) harbored PCV2 DNA, 127 of 212 were positive in Peyer's patches of the ileum (60% prevalence) and 58 of 241 examined fetuses had FISH positive cells (24% prevalence) in the spleen. The prevalence of fetal organ infections and the detailed distribution is shown in table 3. There was a significant difference in the prevalence of PCV2 infection between the investigated primary and secondary lymphatic organs calculated over all groups. Spleen was significantly less often infected than the Peyer's patches of the ileum which was itself significantly less often infected than the mesenteric lymph nodes. However, indeed statistically not calculable due to a zero occurrence of negative infection, thymus was obviously the most infected organ (figure 1).

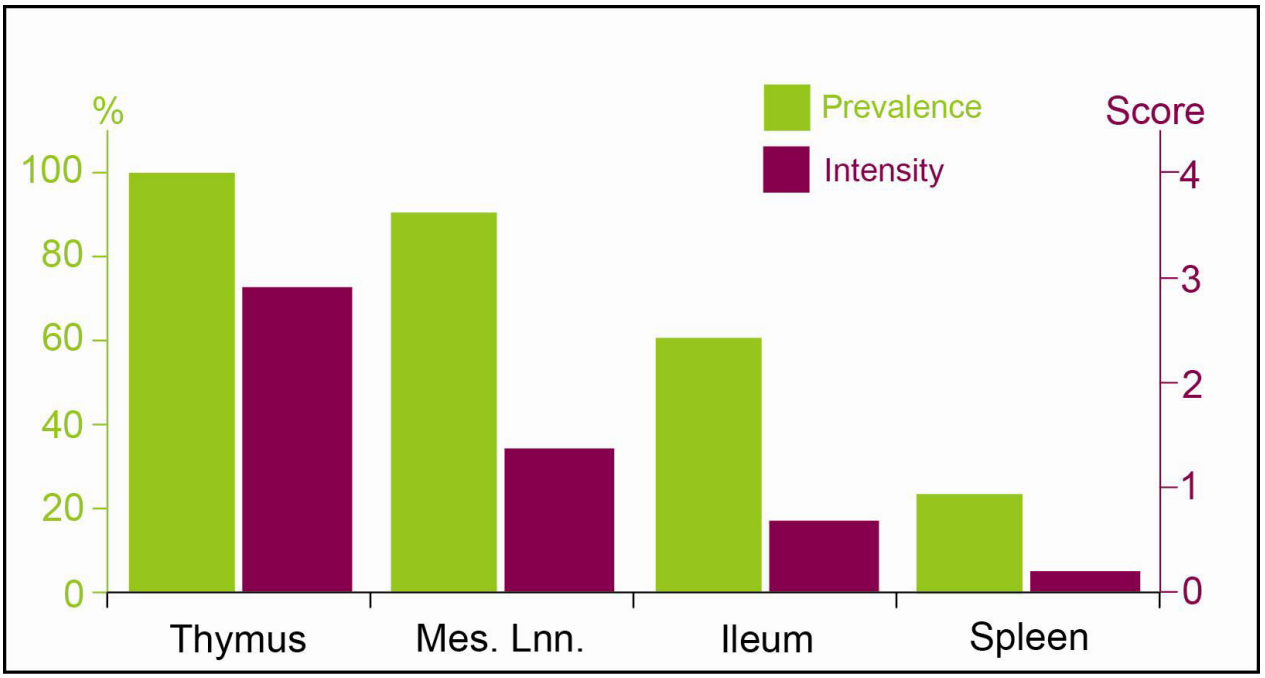

Fig. 1: Prevalence and intensity of PCV2 DNA in thymus, mesenteric lymph nodes, ileum and spleen. We calculated the p-values for intensity between organs with a cut off, which compared not or low infected organs with high infected organs of fetuses. To make the intensity graphically visible we used a score system.

Infection prevalence of an organ was one criteria and virus load in a specific organ was another parameter. To estimate virus load differences of a particular organ between the investigation groups by the categorized data analysis from score 0 to 4, we set a cut off between not or low and higher infected fetuses individually for each organ. Of note, thymus showed no differences in PCV2 DNA load among the control-, the FLEX- and the CVac-group (p=0.1069). Also, there were no significant differences between the control group and the two combined vaccination groups (p=0.6672) considering PCV2-DNA infected thymus cells. However, we found significant differences between the control-, the SVac- and the MVac-group (p=0.0124), with the SVac-group containing less PCV2 DNA positive cells than the other groups. A histogram of the five groups in thymus is shown as an example in figure 2. It was not possible to calculate the significance between the control-, the FLEX- and the CVac-group in mesenteric lymph nodes due to zero degree in the data set. However, table 3 shows a PCV2 infection reduction in the FLEX-group in mesenteric lymph nodes. There was no significant difference between the control group and the both combined vaccination groups

(p=0.3226), but between the control-, the SVac- and the MVac-group (p=0.0027). The SVac-group containes less positive cells in mesenteric lymph nodes then the other groups. Peyer's patches of the ileum from the control group (p=0.0217) were significantly less infected than the Peyer's patches of both combined vaccination groups together. Again, it was not possible to calculate the significance between the control-, the FLEX- and the CVac-group due to zero degree in the data set. The MVac-group (p=0.0326) has significantly more positive cells in Peyer's patches of the ileum then the control- and the SVac-group. Spleen was the lowest infected organ in our investigations. Nevertheless, we found significant differences in the comparisons between the control-, the FLEX- and the CVac-group (p=0.0016), with the CVac-group having less positive cells in the spleen. Also the combined vaccination groups (p=0.0010) have significantly less positive cells in spleen than the control group. The MVac-group (p=0.0008) has significantly less positive cells inside compared to the control- and the SVac-group.

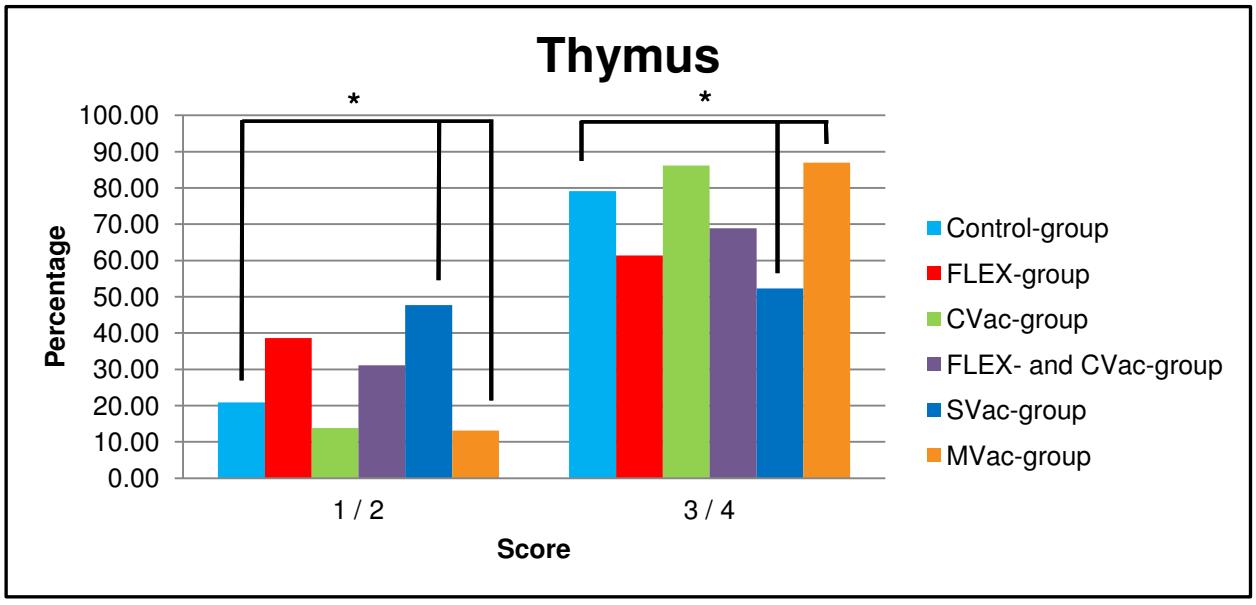

Fig. 2: Comparison of PCV2 DNA intensity between groups of thymal tissues. We compared the groups due to a cut off between low (1 / 2) and high (3 / 4) positive thymal tissues. SVac-group has significantly less high infected thymus (p=0.0124) than control- and MVac-group.

The differences in PCV2 DNA load between the organs were calculated by comparing the highly infected organs between each other. Therefore we set an individual cut off between low and high infection for every single organ. Thymus was not only the most often infected organ, it contained also the highest concentration of PCV2 genomes followed by mesenteric lymph nodes, Peyer's patches of the ileum and spleen. To make this graphically visible we used a score system (figure 1). Though PCV2 DNA is readily measurable, virus antigen was not detected by IHC. This is different in fetuses which died of a PCV2 infection.

Table 3: Frequency and quantity of PCV2 DNA in different fetal tissues.

Tissue	FISH	Control n (%)[a]	FLEX n (%)[a]	CVac n (%)[a]	SVac n (%)[a]	MVac n (%)[a]	Total n (%)[a]
Thymus N=242	0	0	0	0	0	0	0
	1	0	1 (2)	2 (2)	0	3 (3)	3 (1)
	2	19 (21)	21 (37)	11 (12)	21 (48)	11 (10)	51 (21)
	3	59 (65)	35 (61)	66 (70)	23 (52)	78 (73)	160 (66)
	4	13 (14)	0	15 (16)	0	15 (14)	28 (12)
Mesenteric lymph node N=238	0	6 (7)	2 (3)	13 (14)	2 (4)	13 (12)	21 (9)
	1	45 (51)	56 (97)	32 (35)	43 (96)	45 (43)	133 (56)
	2	24 (27)	0	25 (27)	0	25 (24)	49 (21)
	3	13 (15)	0	21 (23)	0	21 (20)	34 (14)
	4	0	0	1 (1)	0	1 (1)	1
Ileum N=212	0	38 (62)	31 (53)	16 (17)	26 (58)	21 (20)	85 (40)
	1	23 (38)	27 (47)	65 (70)	19 (42)	73 (69)	115 (54)
	2	0	0	12 (13)	0	12 (11)	12 (6)
	3	0	0	0	0	0	0
	4	0	0	0	0	0	0
Spleen N=241	0	42 (47)	50 (86)	91 (98)	37 (82)	104 (98)	183 (76)
	1	48 (53)	8 (14)	2 (2)	8 (18)	2 (2)	58 (24)
	2	0	0	0	0	0	0
	3	0	0	0	0	0	0
	4	0	0	0	0	0	0

Tissues of the different organs listed and derived from animals of the control-, FLEX- and CVac-groups were analyzed by FISH and arbitrarily scored from 0-4 for the quantity of PCV2 positive cells present. The vaccinated animals were further grouped as single vaccinated (SVac) or multiple vaccinated (MVac). The total absolute and relative number of events in a given tissue was also calculated (Total).

[a] n(%): absolut and relative number (given as %) of tissues scored as indicated

4.5. Analysis of distribution patterns of FISH positive cells in thymus

In thymus we found two different distribution patterns. Either the positive cells were accumulated in clusters (figure 3a) or they were individually distributed (figure 3b). We always found both patterns in thymus in different proportions. We evaluated the distribution patterns of infected cells by estimating the percentage of positive cells to be present in clusters or individually arranged. The results are shown in table 4. In summary, the FLEX-group (p=0.310) contained significantly higher proportions of infected cells present in clusters than the control-, the CVac-, SVac- or the MVac-group.

Table 4: Frequency and distribution patterns of PCV2 positive cells in thymi.

	Control		FLEX		CVac		SVac[a]		MVac[b]	
Score	Single n (%)	Aggregated n (%)	Single n (%)	Aggregated n (%)	Single n (%)	Aggregated n (%)	Single n (%)	Aggregated n (%)	Single n (%)	Aggregated n (%)
1	0	0	0	1 (2)	0	2 (2)	0	0	0	3 (3)
2	7 (8)	12 (13)	8 (14)	13 (22)	7 (7)	4 (4)	8 (18)	13 (29)	7 (7)	4 (4)
3	36 (40)	23 (25)	4 (7)	31 (53)	41 (44)	25 (27)	3 (7)	20 (44)	42 (39)	36 (34)
4	9 (10)	4 (4)	0	0	6 (6)	9 (10)	0	0	6 (6)	9 (8)
Sub-total	**52 (57)**	**39 (43)**	**12 (21)**	**45 (77)**	**54 (57)**	**40 (43)**	**11 (25)**	**33 (73)**	**55 (51)**	**52 (49)**
Total	91 (100)		57 (98)		94 (100)		44 (98)		107 (100)	

PCV2-positive cells in thymus of animals of the control-, FLEX- or CVac- vaccinated groups appeared as single cells (single) or as cell aggregates (aggregated). The events were arbitrarily scored as absolute (n) and relative (%) numbers as shown in table 3.

[a] SVac: singly vaccinated dams

[b] MVac: dams vaccinated multiple time

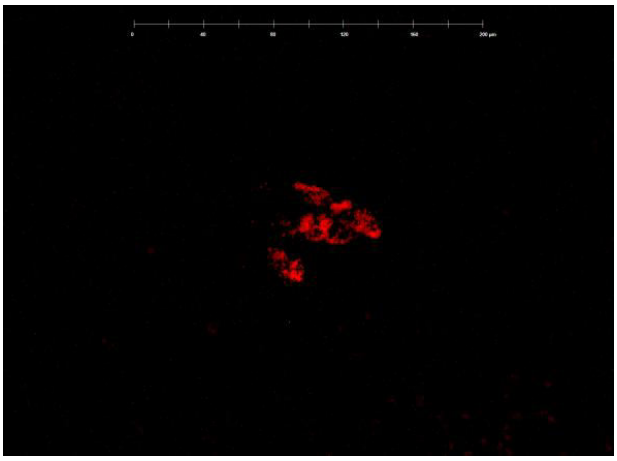

Fig. 3a: PCV2 positive cells in clusters pictured with FISH.

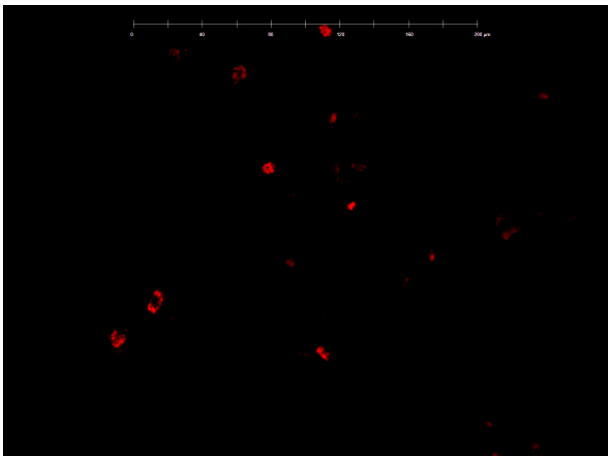

Fig. 3b: PCV2 positive cells arranged free pictured with FISH.

4.6. Existence of replicative forms of PCV2 in thymus

To identify virus propagation potential in the slightly infected fetuses we evaluated some thymus sections for the presence of single and double strand PCV2 genomes by FISH. Indeed, we found double stranded DNA in thymus (figure 4a and 4b) indicating PCV2 propagation in this organ.

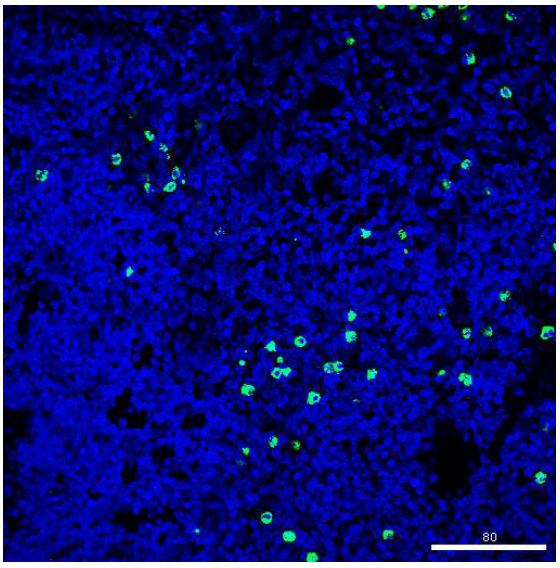

Fig. 4a: The green labeled cells show ssDNA together with dsDNA by FISH.

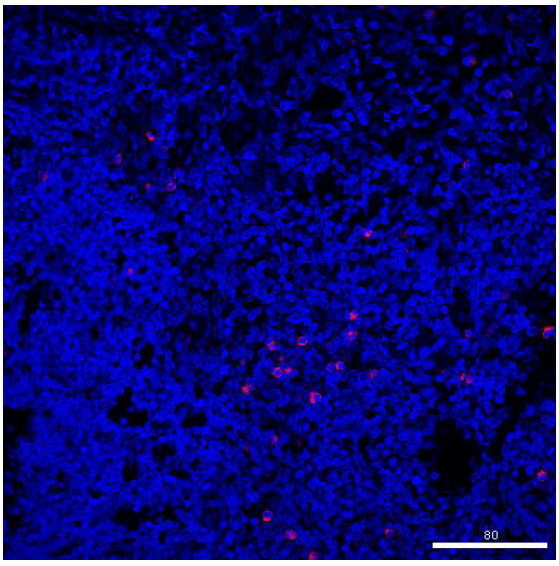

Fig. 4b: In red only dsDNA is visible by FISH.

5. Discussion

Previously, we reported marked improvement of production parameters with vaccination against PCV2 of dams in farms subclinically infected with PCV2 [18]. Additionally, we found fetal thymus of pigs to be persistently, even latently infected with PCV2 DNA (manuscript in preparation). These observations led us to the assumption whether the internal, lymphatic viral pools might be reduced by vaccination and thus, might explain the improvement of production parameters by anti-PCV2 vaccination. We evaluated primary and secondary lymphatic organs from PCV2 IHC negative, immune-competent fetuses by FISH. We detected positive cells in thymus in 100% of the examined fetuses and also in other lymphatic organs but with lower prevalence. Thymus was not only the most often infected organ but also the organ with the highest PCV2 infection density. After vaccination, these PCV2 infection distributions hardly changed in thymus. However, in mesenteric lymph nodes we found a reduced prevalence of PCV2 DNA infection after one vaccination. In thymus a change of infection pattern formation could be found in the FLEX- and the SVac-group. Nevertheless, after multiple vaccinations these internal virus pools leveled out to the levels of fetal organs from non-vaccinated sows in reference to virus prevalence, intensity and pattern formation.

Surprisingly, the thymus is described in PMWS as atrophic and yet, in most studies, thymus was neglected. Exceptions are in animal infection experiments when the organs of the pigs were flooded with PCV2. For example in a study with dams infected intranasally at late gestation, their fetuses harbored PCV2 genomes in thymus [12]. However, these piglets had lesions due to the PCV2 infection and were highly PCV2 antigen positive in the majority of cases. Another recent study detected PCV2 DNA with a prevalence of 39.9% and anti-PCV2 IgG antibodies in 21.4% of neonatal pig blood before their first colostrum intake from clinically normal swine breeding herds [13]. Baker et al. [35] reported a PCV2 prevalence of 0% to 20% in their pigs. However, in this study older sows had more viremic piglets than younger sows. These might be indications that neonates carried PCV2 even before birth.

In contrast, we only detected PCV2 DNA in the blood of seven fetuses (2.9%) and none had a meaningful immune response in form of anti-PCV2 IgM or IgG antibodies. This less than 3% of infected blood from fetuses compared with the 39.9% in the other study

might be accounted to different PCV2 infection pressures on the farms or simply differences in the effectiveness of methodology.

We were not able to detect either anti-PCV2 IgM or anti-PCV2 IgG antibodies in any fetuses. Particularly, we also did not detect PCV2 specific antibodies in fetuses containing PCV2 DNA in serum. Possible reasons for the deficiency of PCV2 specific antibodies could be the low presence or absence of PCV2 capsid protein, or that PCV2 infection occurred before the 57th day of gestation when fetuses are still immune-incompetent. In experimental infections of pregnant dams before the 57th day of gestation, fetuses did not produce any PCV2 specific antibodies [8]. Infections after day 57 induced antibodies in the now immune-competent pig fetuses [8]. These results correlated with another study where other antigens did induce an immune response in pig fetuses only after 58th day of gestation [36]. The argument of the early fetuses' infections with PCV2 gets also supported by the observation that semen [37, 38] or oocytes in the reproduction tract can carry PCV2 [16]. Additionally, PCV2 was able to infect zona pellucida-free morula and early blastocysts [39]. Therefore, PCV2 has the potential to infect embryos in the earliest stages of gravidity. It is also interesting to notice that chicken anemia virus (CAV), a family member of the *Circoviridae* infects chicken oocytes and stays latent until reactivation [27, 40]. Similar to our findings that PCV2 infects the thymus of pig embryos, CAV is also known to infect chicken thymus [29, 41, 42]. Comparable to latent infections with CAV, we did not find any anti-PCV2 antibodies in pig fetuses although we abundantly detected PCV2 genomes and also the replicative PCV2 isoform that makes a low replication of PCV2 plausible. Further investigations are needed to understand more about PCV2 latency and what effect this might have on the health status of individual pigs.

To determine whether sow vaccination might reduce the latent form of PCV2 in fetuses we compared embryos from vaccinated dams to embryos from non-vaccinated mothers. Many reports describe a direct effect of anti-PCV2 immunization of dams or piglets, for example in reduced mortality rate [43] or improved production parameters [44-47]. Subsequently, anti-PCV2 vaccinated dams, after challenge with PCV2, showed reduced PCV2 propagation in utero most probably because of their own reduced PCV2 viremia [19]. However, the vaccination did not prevent fetal PCV2 low-level viremia. Nevertheless, PCV2 antigen was absent in tissues of these neonates [19]. Another report by the same research group suggested that dam-associated PCV2 specific antibodies are not protective for *in utero* PCV2 infection [48]. Although, these data are

very reasonable and important they have to be judged with caution. As these authors were most probably not aware of the latent PCV2 characteristics they additionally infected test animals with PCV2, whereas we compared preexisting endogenous PCV2 levels. We used an advanced oligonucleotide mediated FISH to detect PCV2 DNA in tissues of fetuses. We found no obvious distinction between the control-, the FLEX- and the CVac-group in matters of prevalence and intensity of positive cells in thymus. Statistically significant differences could be found between groups in mesenteric lymph nodes, Peyer's patches of the ileum and spleen. However, pig groups responded to vaccination differently in every organ: for example vaccination had no real beneficial effect for clearing PCV2 infection in Peyer's patches of the ileum. This was different in mesenteric lymph nodes or spleen. In spleen, vaccination in general reduced PCV2 infection and in mesenteric lymph nodes single vaccination was effective in reducing endogenous pre-existing PCV2 concentrations. Interestingly, when we compared pattern formation of PCV2 infected cells of thymus we detected less freely arranged PCV2 infected cells in the FLEX-group. The reduced infectivity of endogenous PCV2 pools related to single versus multiple vaccinations may reflect PCV2 adaptation to host immune responses. That means chronic stimulation with multiple vaccination over several sow generations leaves the virus enough time to conquer back the same space as without vaccination. The single vaccination treatment might simply be a short time of higher immune system pressure on endogenous PCV2 pools.

It is known for herpes sanctuaries to continuously provide new virus [49]. Compared to these we postulate a similar scenario for PCV2 and thymus. For the first time, we could show that PCV2 inherently possesses thymus specificity in fetuses from healthy sows. Furthermore, we could show PCV2 availability of replicative isoform in thymus. This might indicate that PCV2 existence in thymus is based on a low turnover. Thymus belongs to the primary lymphatic system and plays an important role in the development and differentiation of the immune system in fetuses, neonates and developing pigs. The question that immediately comes to mind: what kind of influence have PCV2 infected cells in thymus onto the host immune response and the development of PCVAD?

In conclusion, all fetuses from healthy sows were infected with PCV2 and a vaccination of the dams had no influence on the elimination or reduction of PCV2 load in fetuses. This was the first time where thymus was investigated systematically and found to be the mainly infected organ. On the basis of these results it will be important to continue the research about the influence of PCV2 on T-cell development. Failure to detect anti-

PCV2 antibodies and PCV2 infection before the 57th day of gestation in pig embryos and the analogy to CAV infections make it very plausible that PCV2 infects thymus latently and uses it also as a sanctuary against immune responses.

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